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Immunoproteasome activation enables human TRIM5α restriction of HIV-1

Jose M. Jimenez-Guardeño, Luis Apolonia[†], Gilberto Betancor[†] and Michael H. Malim[*]

Department of Infectious Diseases, School of Immunology & Microbial Sciences, King's College London, London SE1 9RT, UK

[†]These authors contributed equally to this work

*Corresponding author

Department of Infectious Diseases, School of Immunology & Microbial Sciences, King's College London, 2nd Floor Borough Wing, Guy's Hospital, London Bridge, London, SE1 9RT

tel: +44 20 7848 9606

Type 1 interferon (IFN) suppresses viral replication by upregulating the expression of interferon-stimulated genes (ISGs) with diverse anti-viral properties[1]. The replication of human immunodeficiency virus type-1 (HIV-1) is naturally inhibited by IFN, with the steps between viral entry and chromosomal integration of viral DNA being notably susceptible[2-5]. The ISG myxovirus resistance 2 (MX2) has been defined as an effective post-entry inhibitor of HIV-1, but is only partially responsible for IFN's suppressive effect[6-8]. Using siRNA-based library screening in IFNα-treated cells, we sought to characterize further ISGs that target the pre-integration phases of HIV-1 infection, and identified human tri-partite-containing motif 5α (TRIM5α) as a potent anti-HIV-1 restriction factor. Human TRIM5α, in contrast to many non-human orthologues, has not generally been ascribed significant HIV-1 inhibitory function, a finding attributed to ineffective recognition of cytoplasmic viral capsids by TRIM5α[2,9,10]. Here, we demonstrate that IFNα-mediated stimulation of the immunoproteasome, a proteasome isoform mainly present in immune cells and distinguished from the constitutive proteasome by virtue of its different catalytic β-subunits as well as the proteasome activator (PA) 28 regulatory complex[11-13], and the associated accelerated turnover of TRIM5α underpin the reprogramming of human TRIM5α for effective capsid-dependent inhibition of HIV-1 DNA synthesis and infection. These observations identify a mechanism for regulating human TRIM5α anti-viral function in human cells and rationalize how TRIM5α participates in the immune control of HIV-1 infection.

IFNα mobilizes the expression of hundreds of ISGs, with the functions and viral substrates of many awaiting definition[1]. To identify ISGs that suppress HIV-1 replication, we designed an siRNA library targeting 598 ISGs (plus two negative controls; Supplementary Table 1). Focusing

on the early stages of infection (up to and including viral transcription), two cultures of IFNα-responsive U87-MG $CD4^+$ $CXCR4^+$ cells were transfected with each siRNA, with one being maintained with 500 U ml^{-1} IFNα for 24 h and one without. All cultures were then challenged with HIV-1/Nef-internal ribosome entry signal (IRES)-Renilla, a modified replication-competent reporter virus, and infection quantified by measuring Renilla luciferase activity at 48 h (Fig. 1a; unadjusted levels of infection indicated in left hand panel, and folds of IFNα-mediated suppression indicated in right hand panel; Supplementary Fig. 1). Three genes of well-established relevance to HIV-1 infection and whose suppression corresponded with markedly increased levels of infection in the presence of IFNα were interferon regulatory factor 9 (*IRF9*), *MX2* and *TRIM5α* (Supplementary Fig. 2 displays the 14 genes with the strongest effects). IRF9, a transcription factor required for ISG induction[4], and MX2, an established HIV-1 inhibitory ISG[6,7], were anticipated finds, but TRIM5α was completely unexpected. Indeed, human TRIM5α has hithertofore been regarded as being virtually inactive against HIV-1; in contrast, non-human TRIM5α proteins, for example from rhesus macaque, are potent HIV-1 restriction factors that recognize post-entry viral capsids to induce their premature fragmentation and the inhibition of reverse transcription[2,9].

The restorative impact of each component TRIM5α siRNA of the smartpool against IFNα suppressed HIV-1 infection was confirmed using a HIV-1/Nef-IRES-green fluorescent protein (GFP) reporter virus, with IFNα-mediated inhibition consistently decreasing by 2- to 4-fold relative to the non-silencing siControl (Fig. 1b) and TRIM5α expression being reliably silenced (Fig. 1c). Similar levels of rescue of infection were also observed in a bulk cell population and two independent knock-out cell lines in which the endogenous *TRIM5α* alleles had been inactivated using CRISPR-Cas9 genome editing (Fig. 1d and 1e; Supplementary Fig. 3); and across a range

of IFNα doses (Supplementary Fig. 4). Importantly, re-introduction of a CRISPR-resistant human TRIM5α (rTRIM5α) cDNA into these cells completely restored IFNα-mediated suppression, while no changes in HIV-1 susceptibility were detected with an irrelevant control gene (luciferase) (Fig. 1f). shRNA-mediated silencing was then used to suppress TRIM5α expression in primary human CD4+ T cells. Relative to donor matched control samples, reducing TRIM5α levels significantly impeded the IFNα-mediated inhibition of HIV-1 infection, therefore confirming the importance of this IFNα-regulated anti-viral pathway in the principal *in vivo* cell target of HIV-1 infection (Fig. 1g and Supplementary Fig. 5). As noted earlier, it has been established that IFNα is a potent and early inhibitor of HIV-1 reverse transcription following entry into susceptible cells[3]: TRIM5α's substantial contribution to this effect was demonstrated by using quantitative PCR to measure the restoration of viral cDNA accumulation following TRIM5α silencing in IFNα treated cells (~5-fold rescue, Fig. 1h). The broad sensitivity of diverse strains to IFNα-activated human TRIM5α, was confirmed using siRNA-mediated silencing and ten HIV-1 isolates engineered to confer GFP expression following productive infection (extents of rescue ranging from 2- to 5-fold, Supplementary Fig. 6).

TRIM5α-mediated inhibition is initiated through the recognition of viral capsids in the cytoplasm, leading to their fragmentation and the suppression of reverse transcription (viral DNA synthesis). Binding is both sequence- and structure-specific, and is dependent on the capsid lattice (which is composed of multiple hexagonal and pentagonal assemblies the viral Capsid (CA) protein)[14] and the carboxy-terminal SPRY (a sequence repeat in dual-specificity kinase SPlA and RYanodine receptors) domain of TRIM5α[2,9]. The specificity of human TRIM5α for HIV-1 CA was demonstrated by the reduced inhibitory effect of IFNα following TRIM5α silencing that was seen

for virus bearing wild-type HIV-1 CA (WT_{CA}), but not for a matched HIV-1-based chimeric virus carrying (non-recognized) CA derived from a simian immunodeficiency virus (SIV) (SIV_{CA}) (Fig. 2a). The suppression of HIV-1 by human TRIM5α is not, therefore, the result of a generalized induction of TRIM5α anti-lentiviral activity by IFNα.

The central importance of the SPRY domain for TRIM5α function in the presence of IFNα was then confirmed in gene transduction-based experiments using U87-MG $CD4^+$ $CXCR4^+$ cells where endogenous TRIM5α and MX2 expression were ablated using CRISPR-Cas9 genome editing (Fig. 2b; Supplementary Fig. 7 and 8): luciferase and CD8 served as negative controls for not affecting infection (~5-fold suppression of infection in the presence of IFNα), whereas a previously defined TRIM-cyclophilin (TRIMCyp) fusion protein and MX2 were positive controls[6,7,10] whose inhibitory phenotypes do not depend on IFNα (Fig. 2b). Consistent with our findings with the endogenous gene, ectopically expressed human TRIM5α inhibited HIV-1 infection very effectively when IFNα was added (total suppression of ~14-fold). Removing the SPRY domain abolished this effect (~5-fold suppression), whereas including mutations (R332G and R335G) that have previously been shown to confer anti-HIV-1 activity[15,16] still did so to a minor extent and without compromising activation by IFNα (~14-fold total suppression).

TRIM5α is an E3 ubiquitin (Ub) ligase that is polyubiquitinated by cellular E2 Ub conjugating enzymes as a pre-requisite for suppressing reverse transcription and infection[17-19]. Consistent with previous results[20], the integrity of TRIM5α's ligase domain was confirmed as being necessary for viral inhibition through its inactivation by the H43Y mutation (Fig. 2b). Next, to determine the effects of IFNα on TRIM5α ubiquitination and ensuing proteasome-mediated degradation, cells

were co-transfected with vectors expressing FLAG-tagged TRIM5α and HA-tagged Ub, and whole cell lysates or immunoprecipitated FLAG-tagged proteins analyzed by immunoblotting (Fig. 3a). IFNα did not substantially affect global levels of protein ubiquitination (lanes 1 to 8), but did induce a marked decrease in the level of ubiquitinated TRIM5α (lanes 10 and 14). This decrease was reversed by addition of the broadly acting proteasome inhibitor MG132 or the immunoproteasome-specific inhibitor ONX-0914 (lanes 15 and 16, respectively).

Confirmation that IFNα specifically promotes the proteolytic turnover of TRIM5α was obtained in three ways: first, analysis of cells co-expressing FLAG-tagged TRIM5α and FLAG-tagged GFP as a control showed that IFNα induced the marked loss of TRIM5α and that this was blocked by MG132 or ONX-0914 (Fig. 3b); second, levels of endogenous TRIM5α in IFNα treated cells were increased by adding MG132 or ONX-0914 (Fig. 3c; of note, because TRIM5α is an ISG itself its levels were modestly increased by IFNα addition compared to untreated cells[21], despite its accelerated turnover); and third, metabolic-labelling and pulse-chase analysis of FLAG-TRIM5α in transfected cells demonstrated a shortening in initial half-life from ~100 min to ~70 min (Supplementary Fig. 9). As expected for proteasome-mediated degradation[22], blocking the formation of lysine-48-linked polyubiquitin chains by co-expression of the K48R ubiquitin mutant inhibited IFNα-induced TRIM5α proteolysis (Supplementary Fig. 10).

The importance of proteasomal activity for IFNα-mediated suppression was confirmed in viral challenge experiments, where both inhibitors significantly reversed the inhibitory influence of IFNα (~3-fold rescue, Fig. 3d; Supplementary Fig. 11). This effect was shown to be dependent on

TRIM5α function since, in the presence of IFNα, HIV-1 infection of cells lacking endogenous TRIM5α was not improved by MG132 or ONX-0914 (Fig. 3e; Supplementary Fig. 12).

The immunoproteasome is predominantly expressed in cells of haematopoietic origin in response to inflammatory cytokines such as IFN, and differs from the constitutive proteasome with respect to the identity of its three proteolytic β-subunits and dependence upon the PA28 regulatory complex[11,12,23] (Fig. 4a). Its functional divergence from the constitutive proteasome is incompletely understood, though roles in MHC class I antigen processing, T cell differentiation and cytokine modulation have been described[24,25]. In light of the ability of ONX-0914, a selective inhibitor of β5i/PSMB8, to block IFNα-induced TRIM5α function (Fig. 3e), the expression patterns of the immunoproteasome β-subunits and the PA28A/PA28B subunits of the PA28 regulatory complex were analyzed by immunoblotting (Fig. 4b). With some variation, all five proteins accumulated to higher levels in the presence of IFNα.

These five immunoproteasome components, as well as TRIM5α, were then individually silenced using siRNA (Fig. 4b) and the effects on IFNα-induced viral suppression determined (Fig. 4c; Supplementary Fig. 13). All knock-downs conferred a degree of relief from inhibition by IFNα, with PA28A silencing being the most effective to the extent that the magnitude of rescue matched that observed with silencing of TRIM5α itself. Finally, the functional inter-dependence of the IFNα-activated anti-HIV-1 phenotypes of human TRIM5α and the immunoproteasome was established by the substantial diminution in the level of rescue from IFNα inhibition that was observed following PA28A silencing in cells that lacked TRIM5α (Fig. 4d; Supplementary Fig. 14).

TRIM5α restriction has been widely perceived to be important for preventing zoonotic retroviral infections, while being ineffective in controlling viruses in their natural hosts[2,9]. Though there have been sporadic reports of human TRIM5α affecting HIV-1 infection either by suppression of certain HLA-associated CTL escape mutant viruses[26], or by inducing autophagy in Langerhans cells[27], our findings demonstrate broad, non-strain specific inhibition of HIV-1 infection by human TRIM5α. Importantly, we have shown that TRIM5α function is operative in CD4+ T cells, and is dependent on IFNα and activation of the immunoproteasome. Given that IFN levels are elevated during the acute and chronic phases of natural HIV-1 infection[4], we surmise that TRIM5α contributes to the immune control of HIV-1 in infected humans; a conclusion consistent with noted associations between favourable clinical outcomes and elevated TRIM5α expression[28] or specific *TRIM5α* alleles[29,30]. Finally, these results further indicate that the functionality of a specific protein can be fine-tuned by the proteasomal landscape of a cell.

Methods:

HIV-1 molecular clones and retroviral vectors. The NL4-3/Nef-IRES-Renilla and NL4-3/Nef-IRES-green fluorescent protein (GFP) reporter viruses, the HIV-1 based lentiviral vectors containing wild-type Capsid (WT_{CA}) or CA from SIV_{MAC} (SIV_{CA}), and the infectious provirus molecular clones HIV-1 NL4.3, THRO, WITO, CH106.c, REJO.c, SUMA, pAD17, WARO, R66201 and IDU034.2 have been described[31,32,33,34,35].

Cells. U87-MG $CD4^+$ $CXCR4^+$ cells, which stably express the CD4 and CXCR4 receptors have been described[6], and 293T cells were obtained from the American Type Culture Collection

(ATCC). All cell lines were maintained in complete Dulbecco's modified Eagle medium (DMEM)-GlutaMAX (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U ml^{-1} penicillin and 100 g ml^{-1} streptomycin. Human primary $CD4^+$ T cells were isolated from peripheral blood mononuclear cells (PBMCs) of healthy donors (approved by the Guy's Research Ethics Committee; Ref 03/02/06) obtained by density gradient centrifugation through LymphoPrep (Axis-Shield) and isolated by negative selection using the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Cells were activated using Dynabeads Human T-Activator CD3/CD28 (ThermoFisher) and 50 U ml^{-1} recombinant IL-2 (rIL-2) (Roche) for 48 h in RPMI 1640-GlutaMAX medium containing 10% heat-inactivated autologous serum, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin. Cells were maintained after activation in medium containing 30 U ml^{-1} of rIL-2. Cells were treated with IFNα-2b (INTRON A, Merck, Sharpe & Dohme Corp.) for 24 h prior to infection. U87-MG $CD4^+$ $CXCR4^+$ cells were treated with DMSO (Sigma), the proteasome inhibitor MG132 (0.2 μM, Abcam, ab141003) or the immunoproteasome inhibitor ONX-0914 (1 μM, ApexBio, A4011).

siRNA library screen. To identify interferon stimulated genes (ISGs) that modulate HIV-1 infection, we performed an ISGs siRNA screen using a custom siRNA library (Dharmacon) containing 600 siRNA targets (Supplementary Table 1). U87-MG $CD4^+$ $CXCR4^+$ cells were plated at 1.2 x 10^4 per well in a 96-well plate and were reverse transfected with siRNA (10 nM). 24 h later, cells were treated with or without IFNα (500 U ml^{-1}) and, after 12 h incubation, a second siRNA transfection was performed. 24 h after IFNα treatment, cells were challenged with NL4-3/Nef-IRES-Renilla (15 ng $p24^{Gag}$) and infectivity was determined 48 h later by measuring Renilla activity.

Plasmids. pEasiLV plasmids expressing luciferase, CD8, MX2 or TRIMCYP have been described[6]. cDNAs encoding TRIM5α and TRIM5α isoform X7 lacking the SPRY domain were amplified using the SuperScript III One-Step RT–PCR System with Platinum Taq polymerase (Invitrogen) from 20 ng RNA obtained from IFNα-treated (500 U ml^{-1}) U87-MG $CD4^+$ $CXCR4^+$ cells and inserted into pEasiLV-MCS[6] between the AgeI and XhoI restriction sites. The TRIM5α R332G/R335G and H43Y mutants were obtained by site directed mutagenesis (SDM). CRISPR-resistant TRIM5α constructs (rTRIM5α) were produced by SDM using primers designed to silently mutate the guide RNA target sequence. pFLAG-TRIM5α was generated by subcloning a cDNA encoding TRIM5α with an N-terminal FLAG tag into pCAGGS (Addgene) using the EcoRI and XhoI sites. pCAGGS expressing FLAG-tagged GFP, pFLAG-GFP, was derived from a plasmid encoding HA-tagged GFP[36]. The vectors encoding HA-tagged ubiquitin and the K48R and K63R mutant derivatives have been described[37 38].

Knock-out cells. To generate CRISPR/Cas9 cells, sequences encoding specific guide RNAs targeting TRIM5α, MX2 or red fluorescent protein (RFP), as a control, were cloned into BsmBI-linearized lentiviral vector pLentiCRISPRv2[39,40] using the oligonucleotides (forward/reverse) 5′-caccgCCTCCTCCTTTACATTAACC-3′ and 5′-aaacGGTTAATGTAAAGGAGGAGGc-3′ for TRIM5α, (5′-caccgGACAACCAGCCCCGAGACAT-3′ and 5′-aaacATGTCTCGGGGCTGGTTGTCc-3′) for MX2 or 5′-caccgCTCAGTTCCAGTACGGCTCCA-3′ and 5′-aaacTGGAGCCGTACTGGAACTGAGc-3′ for RFP. Vectors expressing the indicated guide RNAs were produced by co-transfection of 293T cells with pLentiCRISPRv2, p8.91 and VSV-G vectors at a ratio of 1.5:1.5:0.375, respectively. U87-MG $CD4^+$ $CXCR4^+$ cells were transduced with the TRIM5α targeting or RFP control lentiviral vectors and selected using 1 μg ml^{-1} puromycin for three days. Single-cell clones

(TRIM5α #1 and TRIM5α #2) were derived from the bulk (TRIM5α) population by single-cell sorting in 96-well plates and assayed for the loss of TRIM5α by immunoblotting. TRIM5α gene disruption was validated by PCR amplification and sequencing of the targeted genomic region. To generate TRIM5α/MX2 double knock-out cells, the TRIM5α #1 line (Fig. 1d) was further transduced with a high concentration of the MX2 targeting vector and the loss of MX2 expression in the bulk population was confirmed by immunoblotting.

Viral production. All viruses were generated by transfection of 10 cm 293T cultures using TransIT-2020 transfection reagent (Mirus), the medium was changed after 6 h and virus containing medium was filtered and collected 48 h after transfection. NL4-3/Nef-IRES-GFP and NL4-3/Nef-IRES-Renilla viral particles were produced by transfection of 10 µg of proviral plasmid. For analysis of HIV-1 reverse transcription products and shRNA-mediated TRIM5α silencing, virus containing supernatants were DNAse (RQ1 RNase free DNAse; Promega) treated for 1 h at 37 °C, and viruses were purified through a 20% (wt/vol) sucrose cushion at 145,370 × g for 75 min at 4 °C, re-suspended in phosphate buffered saline (PBS) and stored in aliquots at -80 °C. Lentiviral vectors stocks were produced by co-transfection of 293T cells with plasmids encoding Gag-Pol (p8.91 or p8.91-SIV_{CA}), a mini viral genome bearing a CMV-GFP expression cassette (pRRL.sin.cPPT.CMV/eGFP), and the VSV-G envelope (pMD.G), at a ratio of 4:4:2, respectively[3]. To generate GFP encoding derivatives of full-length molecular clones NL4.3, THRO, WITO, CH106.c, REJO.c, SUMA, pAD17, WARO, R66201 and IDU034.2 provirus plasmids were co-transfected with the GFP reporter vector pCSGW and VSV-G at a ratio of 4.5:3:3, respectively[41]. Virus titers were determined by challenging U87-MG $CD4^+$ $CXCR4^+$ cells with different viral dilutions and by quantifying the number of GFP-positive cells after 48 h by flow cytometry (FACSCalibur; BD Biosciences) (a typical example is displayed in Supplementary

Fig. 15), and $p24^{Gag}$ content was quantified using an HIV-1 p24 ELISA detection kit (Perkin-Elmer). To produce EasiLV particles, 293T cells were co-transfected with pEasiLV, p8.91, pptTRKrab and VSV-G at a ratio of 1:1:0.5:0.25, respectively[6]. 48 h after transfection, viral particles were harvested, filtered, and used directly to transduce U87-MG $CD4^+$ $CXCR4^+$ cells. 6 h after transduction, the medium was replaced with fresh medium containing 0.5 μg ml^{-1} doxycycline (Sigma) to induce transgene expression. 72 h after transduction, the percentage of E2-Crimson-positive cells was scored (typically >80%) by flow cytometry.

shRNA-mediated TRIM5α silencing. TRIM5α silencing in primary human $CD4^+$ T cells was achieved using a modified version of the HIV-1-based lentiviral vector pHRSIREN-S-SBP-ΔLNGFR-W[42] where the selectable marker for antibody-free magnetic cell sorting sequence (SBP-ΔLNGFR) was replaced by the E2-Crimson fluorescent reporter gene for flow cytometry analysis, generating the pHRSIREN-S-E2-W vector. The shRNA targeting sequence for TRIM5α[43] (shTRIM5) was 5′-TGGCTTCTGGAATCCTGGTTAA-3′ and the scrambled shRNA sequence[42] (shControl) used as negative control was 5′-GTTATAGGCTCGCAAAAGG-3′. Lentiviral vectors were produced by co-transfection of 293T cells with p8.91, VSV-G and the pHRSIREN-S-E2-W vectors at a ratio of 1:0.5:1.5, respectively. Lentiviral particles were concentrated by ultracentrifugation prior to use for transduction of primary human $CD4^+$ T cells at 2000 x g for 2 h. 48 h after transduction, E2-crimson-positive cells were sorted by flow cytometry (typically >90%) and cultured in fresh medium. When indicated, 2.5 x 10^4 cells per well in 96-well plates were treated with IFNα (2000 U ml^{-1}) for 24 h before infection with NL4-3/Nef-IRES-Renilla (30 ng $p24^{Gag}$) at 2000 x g for 2 h and infectivity was measured 48 h after infection by measuring Renilla activity.

Quantification of mRNA expression. 10^6 activated primary human $CD4^+$ T cells were collected with or without 24 h treatment with IFNα, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). cDNA was generated using 500 ng RNA and the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher) and qPCR of selected genes was performed using the following TaqMan gene expression assays (ThermoFisher): TRIM5α (Hs01552559_m1), MX2 (Hs01550808_m1) and GAPDH (Hs99999905_m1). Expression levels of target genes were normalized to GAPDH.

Measurement of HIV-1 reverse transcription products. 10^5 U87-MG $CD4^+$ $CXCR4^+$ were reverse transfected with 10 nM of a smartpool siRNA targeting human TRIM5α or a non-targeting siRNA (Dharmacon). Cells were treated with or without 500 U ml^{-1} IFNα and transfected again 12 h after treatment. 24 h after IFNα treatment, cells were challenged with NL4-3/Nef-IRES-GFP (20 ng $p24^{Gag}$) for 2 h and 48 h after infection, cells were collected and total DNA extraction was performed using the DNeasy kit (Qiagen). Strong-stop cDNA products were detected using primers that amplify the regions between nucleotides 500 and 635 of the provirus: oHC64, (5'-TAACTAGGGAACCCACTGC) and oHC65 (5'-GCTAGAGATTTTCCACACTG) with probe oHC66 (5'-FAM-ACACAACAGACGGGCACACACTA-TAMRA), where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine. qPCR reactions were performed in triplicate using TaqMan Universal PCR master mix, 900 nM of each primer, and 250 nM of probe. After 10 min at 95 °C, reactions were cycled through 15 s at 95 °C, followed by 1 min at 60 °C for 40 repeats, carried out using the QuantStudio 5 Real-Time PCR System (Applied Biosystems). NL4-3/Nef-IRES-GFP plasmid was diluted in salmon sperm DNA solution (20 ng ml^{-1}) to create dilution standards to calculate relative cDNA copy numbers and confirm the linearity of the assays.

siRNA mediated knock-down. U87-MG $CD4^+$ $CXCR4^+$ cells were plated at 1.2 x 10^4 per well in a 96-well plate and were reverse transfected with 10 nM of smartpool siRNAs (Dharmacon) targeting TRIM5α (M-007100-00-0005), PSMB8 (M-006022-01-0005), PSMB9 (M-006023-02-0005), PSMB10 (M-006019-01-0005), PA28A (M-012254-00-0005), PA28B (M-011370-00-0005) or individual siRNAs targeting TRIM5α (D-007100-01-0002, D-007100-02-0002, D-007100-03-0002, D-007100-04-0002). A non-targeting siRNA (D-001210-03-05) was used as a negative control. 24 h after the first transfection, cells were treated with or without IFNα (500 U ml^{-1}) and a second siRNA transfection was performed 12 h after treatment. 24 h after IFNα treatment, cells were challenged with the indicated viruses and infectivity was determined 48 h later. To confirm knock-down efficiency, cell lysates were harvested 24 h after IFNα treatment and were subjected to SDS-PAGE and immunoblot assays.

Immunoblotting. Cells were washed with PBS, lysed in sample buffer, boiled for 3 min, resolved by SDS-PAGE and analyzed by immunoblotting using primary antibodies specific for human MX2 (sc-47197 (N-17), Santa Cruz Biotechnology), human TRIM5α (ab4389, Abcam), human PSMB8 (ab3329, Abcam), human PSMB9 (ab3328, Abcam), human PSMB10 (ab77735, Abcam), human PA28A (ab155091, Abcam), human PA28B (ab183727, Abcam), FLAG (HRP-conjugated M2, Sigma), HA (HRP-conjugated 3F10, Sigma) or human α-tubulin (DM1A, Sigma), and detected using either horseradish peroxidase-conjugated secondary antibodies and chemiluminescence (ECL^+ western blotting substrate, Pierce) or IRDye®-800CW-labelled secondary antibodies and the LI-COR infrared imaging technology (LI-COR UK LTD).

Immunoprecipitation 3 x 10^6 U87-MG $CD4^+$ $CXCR4^+$ cells were seeded in 10 cm tissue culture plates and transfected with the indicated plasmids expressing FLAG-TRIM5α and/or HA-ubiquitin at a ratio of 1:0.5, using TransIT-2020 transfection reagent according to manufacturer's instructions. After 24 h, indicated cells were treated with fresh medium containing or not IFNα (500 U ml^{-1}) and DMSO, MG132 (0.2 μM) or ONX-0914 (1 μM). 24 h after treatment, cells were washed twice with PBS, harvested with lysis buffer (1x PBS, 0.5% Triton X-100 and protease inhibitor cocktail (Roche)) and disrupted by brief sonication. Lysates were cleared by centrifugation at 10000 × g for 10 min at 4 °C and FLAG-TRIM5α was immunoprecipitated using anti-FLAG M2 magnetic beads (M8823-5ML, Sigma) for 2 h at 4 °C and the beads washed a further 4 times in lysis buffer before adding sample buffer (200 mM Tris-HCl pH 6.8, 5.2% SDS, 20% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol). HA-tagged proteins (ubiquitinated proteins) were resolved by SDS-PAGE and detected by immunoblotting using HRP-conjugated anti-HA antibodies.

S^{35} pulse-chase labelling and radio-immunoprecipitation. U87-MG $CD4^+$ $CXCR4^+$ cells were seeded at 2 x 10^5 per well in 6-well plates and transfected with pFLAG-TRIM5α using TransIT-2020 transfection reagent according to the manufacturer's instructions. After 24 h, indicated cells were treated with fresh medium containing or not IFNα (500 U ml^{-1}). 24 h later, cells were washed twice with PBS and incubated for 20 min in cysteine/methionine depleted DMEM (Gibco) at 37°C. The medium was replaced by depletion medium containing 0.25 mCi ml^{-1} S^{35}-labelled cysteine/methionine and the cells maintained for 10 min at 37°C. Cells were then washed twice with PBS and incubated in DMEM. The cells were harvested at various times with lysis buffer (1x PBS, 0.5% Triton X-100 and protease inhibitor cocktail), incubated on ice for 10 min and clarified by centrifugation at 10000 × g for 10 min at 4 °C. FLAG-TRIM5α was immunoprecipitated using

anti-FLAG M2 magnetic beads, resolved by SDS-PAGE and exposed on a phosphor image screen over several days before development on a Typhoon Trio phosphorimager (GE Healthcare).

Statistical analysis. Results in bar charts are expressed as means ± standard deviation (s.d.) for experimental replicates in each case. Differences between the experimental groups were evaluated, where indicated, by paired or unpaired two-tailed *t*-tests.

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

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Author Contributions J.M.J.-G, L.A. and M.H.M. conceived the siRNA screen; J.M.J.-G and M.H.M. designed the study and wrote the manuscript with input from all co-authors; J.M.J.-G carried out the experiments and analyzed the data; L.A. and G.B. contributed to the execution of experiments and provided reagents; and, M.H.M. supervised all aspects of the project.

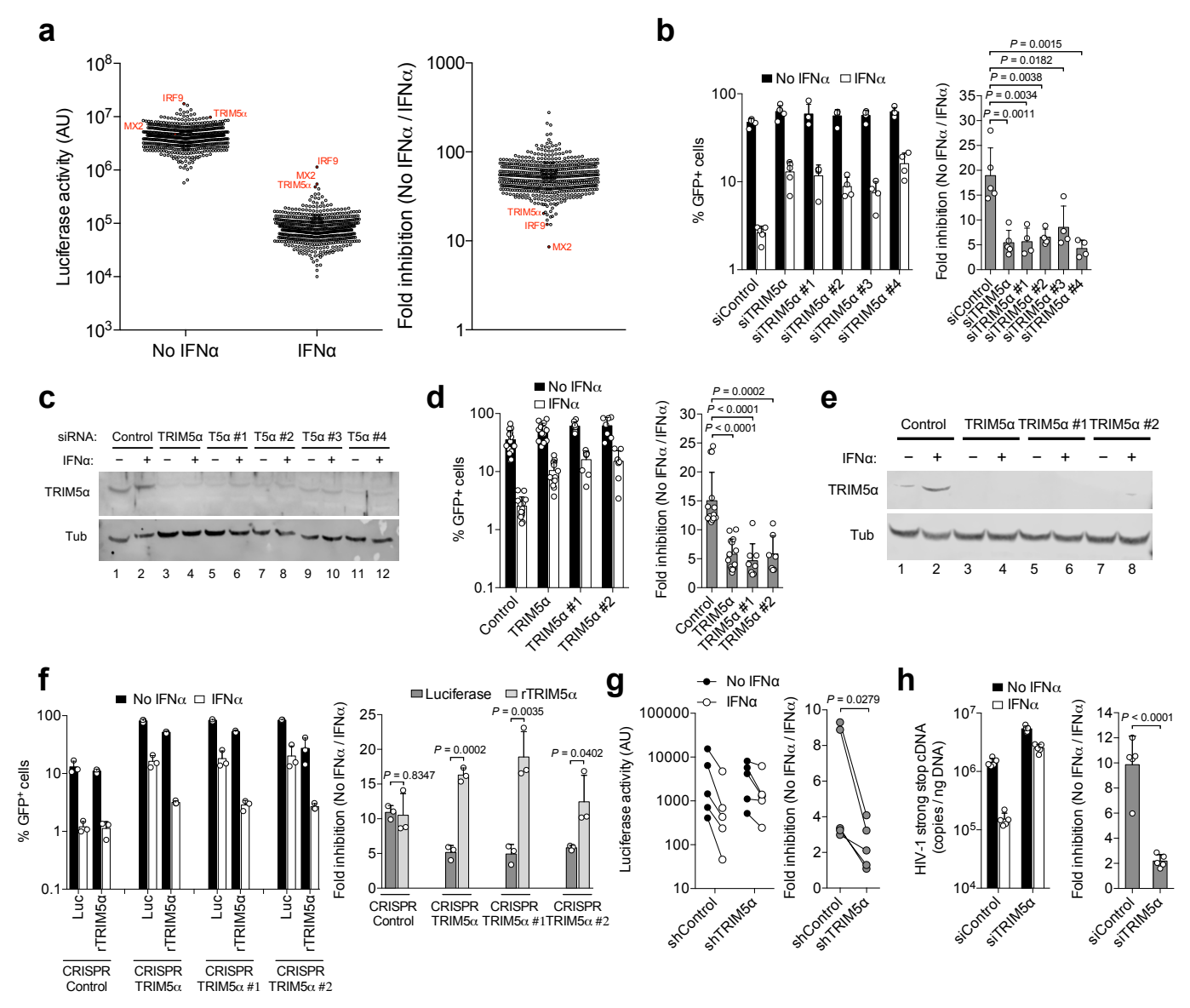
Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.H.M. (michael.malim@kcl.ac.uk)

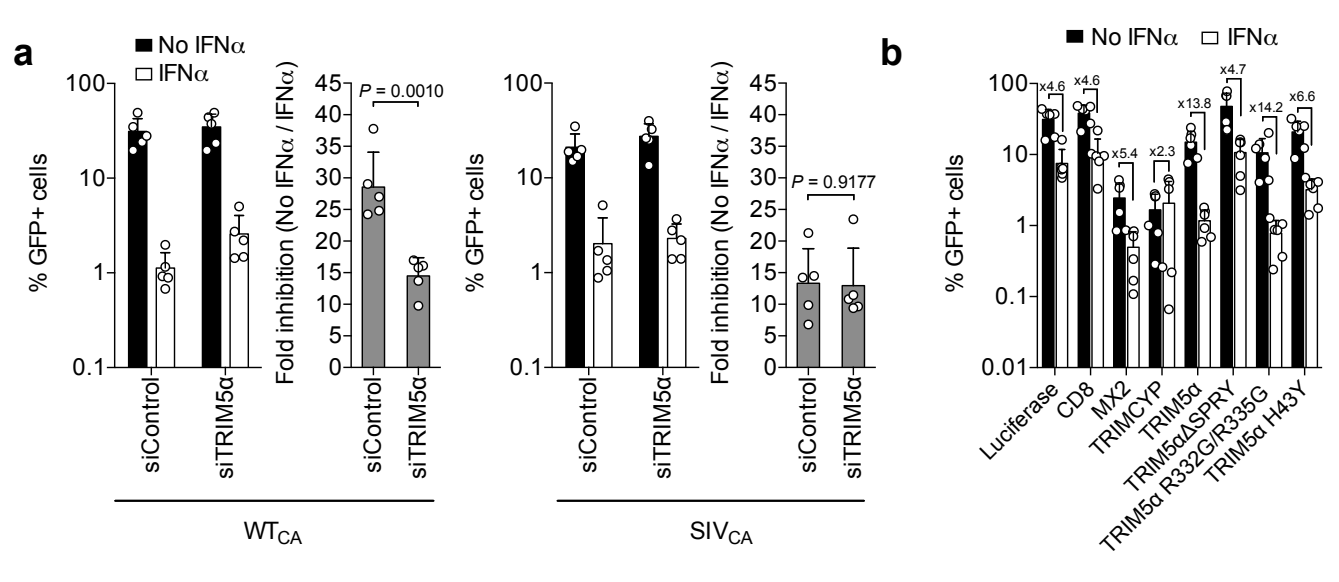
Figure 1. Human TRIM5α is a key effector in the interferon-induced suppression of HIV-1 infection. a, Dot plots of NL4-3/Nef-IRES-Renilla infectivity and IFNα-induced fold inhibition at 48 h post-infection in U87-MG $CD4^+$ $CXCR4^+$ cells doubly transfected with siRNAs targeting 598 ISGs and 2 negative controls with or without the addition of 500 U ml^{-1} IFNα for 24 h. Three notably influential ISGs are indicated in red. **b,** Percentage of GFP-positive cells and IFNα-induced fold inhibition in U87-MG $CD4^+$ $CXCR4^+$ cells infected with NL4-3/Nef-IRES-GFP after TRIM5α silencing using SMARTpool (n = 5) or individual siRNAs (n = 4) with or without added 500 U ml^{-1} IFNα. **c,** Immunoblot analysis of TRIM5α expression in U87-MG $CD4^+$ $CXCR4^+$ cells after siRNA transfection, α-tubulin served as a loading control. One representative immunoblot from two independent experiments is shown. **d,** NL4-3/Nef-IRES-GFP infection and IFNα-induced inhibition in U87-MG $CD4^+$ $CXCR4^+$ bulk [TRIM5α (n = 14)] and clonal [TRIM5α #1 (n = 8) and #2 (n = 7)] cell lines transduced to express TRIM5α specific guide RNAs, with or without added 500 U ml^{-1} IFNα. CRISPR/Cas9 control cells expressed an unrelated guide RNA (n = 14). **e,** Ablation of TRIM5α expression in CRISPR/Cas9 engineered U87-MG $CD4^+$ $CXCR4^+$ cells was verified by immunoblotting, α-tubulin served as a loading control. One representative immunoblot from three independent experiments is shown. **f,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection in TRIM5α deficient U87-MG $CD4^+$ $CXCR4^+$ cells (CRISPR TRIM5α, TRIM5α #1 and TRIM5α #2) or cells expressing an unrelated guide RNA (CRISPR control) transduced with EasiLV lentivirus vectors expressing luciferase (Luc) or a CRISPR-resistant TRIM5α (rTRIM5α) with or without added 500 U ml^{-1} IFNα (n = 3). **g,** NL4-3/Nef-IRES-Renilla infectivity and IFNα-induced inhibition at 48 h post-infection in primary human CD4+ T cells transduced with shRNAs targeting TRIM5α or a control shRNA, and treated with or without 2000 U ml^{-1} IFNα for 24 h prior to infection (n = 5). **h,** U87-MG $CD4^+$ $CXCR4^+$ cells were transfected with control or TRIM5α-specific siRNAs and treated or not with 500 U ml^{-1} IFNα for 24 h before 2 h infections with NL4-3/Nef-IRES-GFP (corresponding to 20 ng $p24^{Gag}$). DNA was harvested at 48 h post-infection and early reverse transcription products (strong stop) and IFNα-induced inhibition were determined by qPCR (n = 5). Data are represented as the mean ± s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired (b, d, f and h) or paired (g) t-tests.

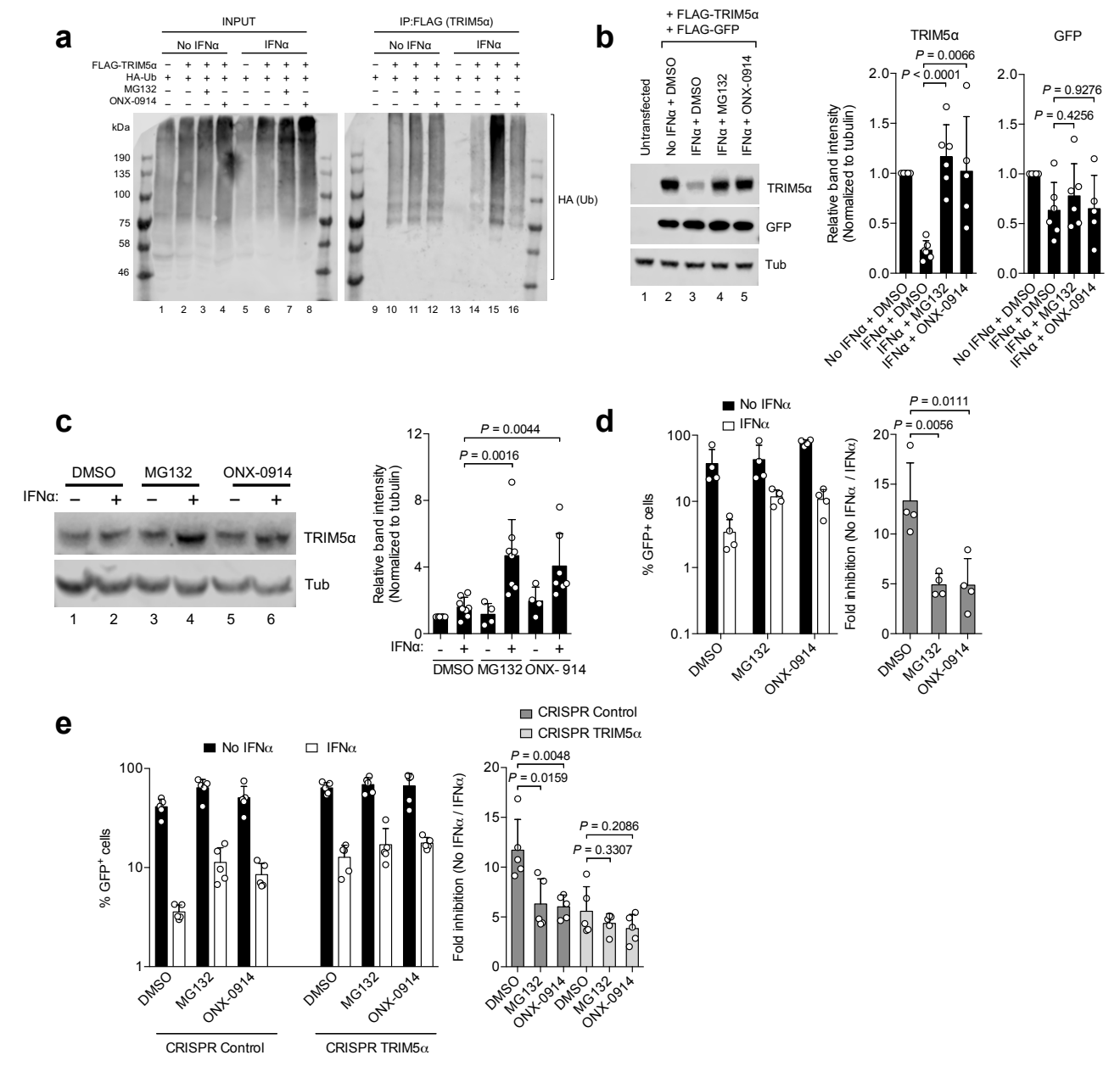
Figure 2. HIV-1 Capsid determines IFNα-induced restriction by human TRIM5α. a, Percentage of GFP-positive cells and IFNα-induced inhibition at 48 h post-infection in U87-MG $CD4^+$ $CXCR4^+$ cells challenged with GFP-encoding HIV-1-based vectors containing wild-type CA (WT_{CA}) or CA from SIV_{MAC} (SIV_{CA}). TRIM5α was silenced using SMARTpool siRNAs and cells treated with or without 500 U ml^{-1} IFNα before infection (n = 5). *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests. **b,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection in TRIM5α/MX2 doubly deficient U87-MG $CD4^+$ $CXCR4^+$ cells (Supplementary Fig. 7) transduced with EasiLV lentivirus vectors expressing luciferase, CD8, CRISPR-resistant MX2, TRIMCYP or CRISPR-resistant TRIM5α, TRIM5αΔSPRY, TRIM5α R332G/R335G or TRIM5α H43Y with or without added 500 U ml^{-1} IFNα (n = 6). Numbers represent fold inhibition of HIV-1 infection after IFNα treatment. Data are represented as the mean ± s.d.

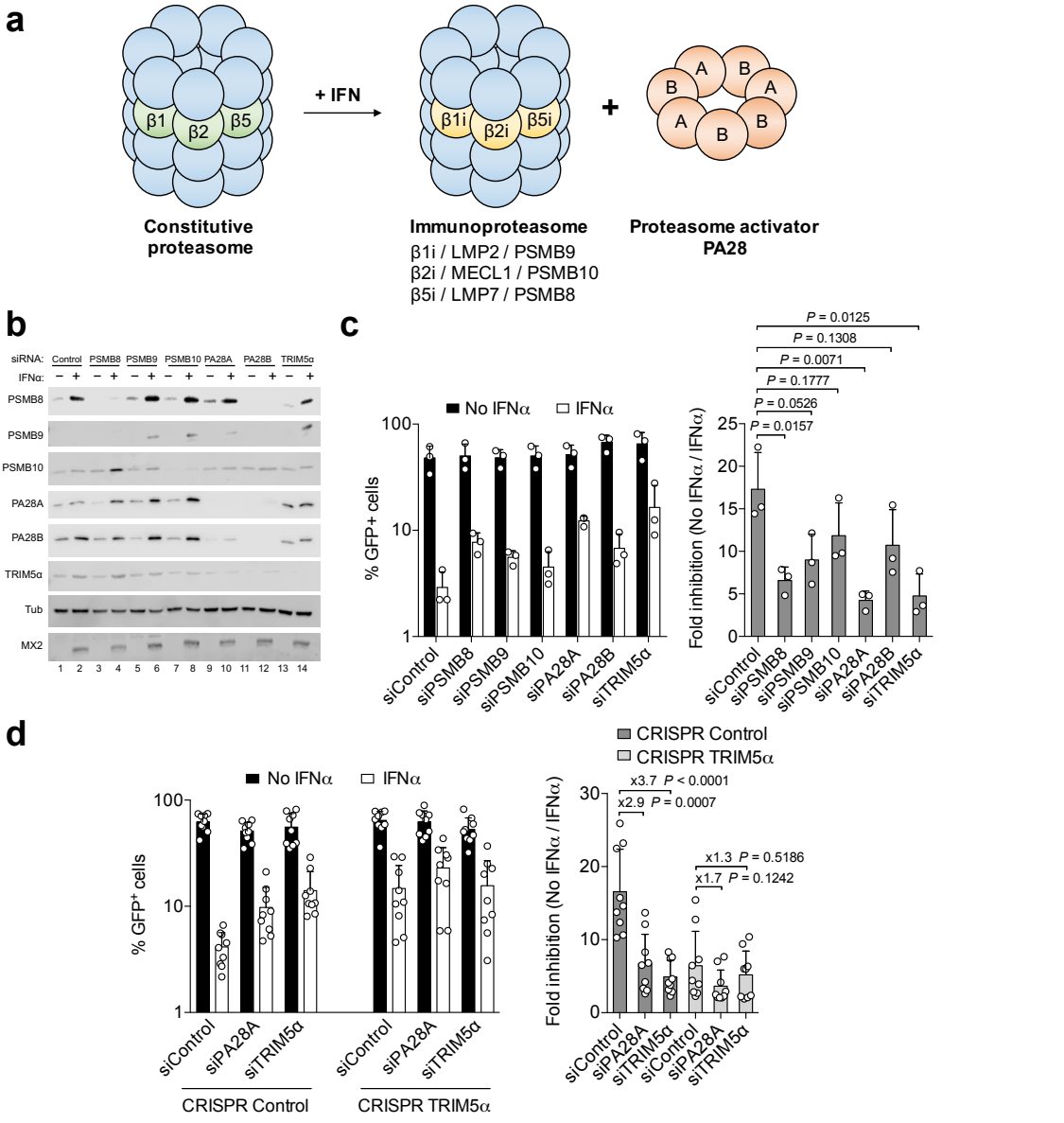
Figure 3. HIV-1 restriction by human TRIM5α requires a functional proteasome system. a, U87-MG $CD4^+$ $CXCR4^+$ cells were co-transfected with a vector expressing HA-ubiquitin[37] and pFLAG-TRIM5α or a control and treated with or without 500 U ml^{-1} IFNα for 24 h in presence of DMSO (negative control), the proteasome inhibitor MG132 (0.2 μM), or the immunoproteasome inhibitor ONX-9014 (1 μM). Whole cell lysates and immunoprecipitated TRIM5α were analyzed by immunoblotting using an anti-HA (ubiquitin) antibody. One representative immunoblot from three independent experiments is shown. **b,** U87-MG $CD4^+$ $CXCR4^+$ cells were co-transfected with pFLAG-TRIM5α and pFLAG-GFP. 24 h after transfection, cells were treated with or without 500 U ml^{-1} IFNα for 24 h in the presence of DMSO (n = 6), MG132 (n = 6) or ONX-0914 (n = 5), lysed and analyzed by immunoblotting using antibodies specific for TRIM5α or FLAG. α-tubulin served as a loading control. The bar graphs represent the relative band intensities for each protein normalized to α-tubulin. **c,** U87-MG $CD4^+$ $CXCR4^+$ cells were cultured with or without 500 U ml^{-1} IFNα, then for with DMSO (n = 8), MG132 (n = 4 without IFNα and n = 8 with IFNα) or ONX-9014 (n = 4 without IFNα and n = 7 with IFNα) for 24 h and endogenous TRIM5α levels were determined by immunoblotting. α-tubulin served as a loading control. The bar graph represents the relative band intensities for TRIM5α normalized to α-tubulin. **d,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection in U87-MG $CD4^+$ $CXCR4^+$ cells with or without added 500 U ml^{-1} IFNα for 24 h before infection in the presence of DMSO, MG132, or ONX-0914 (n = 4). **e,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection in U87-MG $CD4^+$ $CXCR4^+$ cells expressing an unrelated guide RNA (CRISPR control) or specific guide RNAs targeting TRIM5α (CRISPR TRIM5α; bulk Figure 1d) treated with or without 500 U ml^{-1} IFNα in the presence of DMSO, MG132 or ONX-0914 prior to infection (n = 5). Data are represented as the mean ± s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests.

Figure 4. HIV-1 restriction by human TRIM5α is regulated by the immunoproteasome. a, Upon IFNα stimulation, the catalytic subunits (β1, β2, and β3) of the constitutive proteasome are replaced by inducible catalytic subunits (β1i, β2i and β5i) and expression of the proteasome activator PA28 subunits A and B is induced. **b,** Protein levels of immunoproteasome components and PA28 subunits in U87-MG $CD4^+$ $CXCR4^+$ cells after siRNA transfection with or without 500 U ml^{-1} IFNα were determined by immunoblotting. α-tubulin served as a loading control. Immunoblot analysis of the panel was performed once in entirety. **c,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection in U87-MG $CD4^+$ $CXCR4^+$ cells after siRNA-mediated silencing of the indicated genes with or without addition of 500 U ml^{-1} IFNα 24 h before infection (n = 3). **d,** NL4-3/Nef-IRES-GFP infectivity and IFNα-induced inhibition at 48 h post-infection after siRNA-mediated silencing of PA28A or TRIM5α in U87-MG $CD4^+$ $CXCR4^+$ cells expressing an unrelated guide RNA (CRISPR control) or specific guide targeting TRIM5α (CRISPR TRIM5α). 500 U ml^{-1} IFNα was added for 24 h before infection (n = 9). Numbers represent changes in the folds of inhibition. Data are represented as the mean ± s.d. *P* values (95% confidence interval) were determined using two-sided, unpaired t-tests.


a
Luciferase activity (AU)
IRF9
TRIM5α
MX2
No IFNα
IFNα
Fold inhibition (No IFNα / IFNα)
b
No IFNα
IFNα
% GFP+ cells
siControl
siTRIM5α
siTRIM5α #1
siTRIM5α #2
siTRIM5α #3
siTRIM5α #4
Fold inhibition (No IFNα / IFNα)
P = 0.0015
P = 0.0182
P = 0.0038
P = 0.0034
P = 0.0011
c
siRNA:
Control
TRIM5α
T5α #1
T5α #2
T5α #3
T5α #4
IFNα:
TRIM5α
Tub
d
No IFNα
IFNα
% GFP+ cells
Control
TRIM5α
TRIM5α #1
TRIM5α #2
Fold inhibition (No IFNα / IFNα)
P = 0.0002
P < 0.0001
P < 0.0001
e
Control
TRIM5α
TRIM5α #1
TRIM5α #2
IFNα:
TRIM5α
Tub
f
No IFNα
IFNα
% GFP+ cells
Luc
rTRIM5α
CRISPR Control
CRISPR TRIM5α
CRISPR TRIM5α #1
CRISPR TRIM5α #2
Luciferase
rTRIM5α
Fold inhibition (No IFNα / IFNα)
P = 0.8347
P = 0.0002
P = 0.0035
P = 0.0402
g
No IFNα
IFNα
Luciferase activity (AU)
shControl
shTRIM5α
Fold inhibition (No IFNα / IFNα)
P = 0.0279
h
No IFNα
IFNα
HIV-1 strong stop cDNA (copies / ng DNA)
siControl
siTRIM5α
Fold inhibition (No IFNα / IFNα)
P < 0.0001


a
No IFNα
IFNα
% GFP+ cells
100
10
1
0.1
siControl
siTRIM5α
Fold inhibition (No IFNα / IFNα)
45
40
35
30
25
20
15
10
5
0
P = 0.0010
WTCA
P = 0.9177
SIVCA
b
No IFNα
IFNα
% GFP+ cells
0.01
x4.6
x4.6
x5.4
x2.3
x13.8
x4.7
x14.2
x6.6
Luciferase
CD8
MX2
TRIMCYP
TRIM5α
TRIM5αΔSPRY
TRIM5α R332G/R335G
TRIM5α H43Y


a
INPUT
IP:FLAG (TRIM5α)
No IFNα
IFNα
FLAG-TRIM5α
HA-Ub
MG132
ONX-0914
kDa
190
135
100
75
58
46
HA (Ub)
1 2 3 4 5 6 7 8
9 10 11 12 13 14 15 16
b
+ FLAG-TRIM5α
+ FLAG-GFP
Untransfected
No IFNα + DMSO
IFNα + DMSO
IFNα + MG132
IFNα + ONX-0914
TRIM5α
GFP
Tub
1 2 3 4 5
TRIM5α
GFP
Relative band intensity (Normalized to tubulin)
P = 0.0066
P < 0.0001
P = 0.9276
P = 0.4256
c
DMSO
MG132
ONX-0914
IFNα:
TRIM5α
Tub
1 2 3 4 5 6
Relative band intensity (Normalized to tubulin)
P = 0.0044
P = 0.0016
DMSO MG132 ONX- 914
d
No IFNα
IFNα
% GFP+ cells
Fold inhibition (No IFNα / IFNα)
P = 0.0111
P = 0.0056
e
No IFNα
IFNα
% GFP+ cells
CRISPR Control
CRISPR TRIM5α
Fold inhibition (No IFNα / IFNα)
P = 0.0048
P = 0.0159
P = 0.2086
P = 0.3307


a
β1
β2
β5
+ IFN
β1i
β2i
β5i
+
A
B
Constitutive proteasome
Immunoproteasome
β1i / LMP2 / PSMB9
β2i / MECL1 / PSMB10
β5i / LMP7 / PSMB8
Proteasome activator PA28
b
siRNA: Control PSMB8 PSMB9 PSMB10 PA28A PA28B TRIM5α
IFNα: − + − + − + − + − + − + − +
PSMB8
PSMB9
PSMB10
PA28A
PA28B
TRIM5α
Tub
MX2
1 2 3 4 5 6 7 8 9 10 11 12 13 14
c
No IFNα
IFNα
% GFP+ cells
100
10
1
siControl
siPSMB8
siPSMB9
siPSMB10
siPA28A
siPA28B
siTRIM5α
Fold inhibition (No IFNα / IFNα)
P = 0.0125
P = 0.1308
P = 0.0071
P = 0.1777
P = 0.0526
P = 0.0157
25
20
15
10
5
0
d
No IFNα
IFNα
% GFP+ cells
100
10
1
siControl
siPA28A
siTRIM5α
CRISPR Control
CRISPR TRIM5α
CRISPR Control
CRISPR TRIM5α
Fold inhibition (No IFNα / IFNα)
x3.7 P < 0.0001
x2.9 P = 0.0007
x1.3 P = 0.5186
x1.7 P = 0.1242
30
20
10
0